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# Impact of Competitive Inhibition and Sequence Variation upon the Sensitivity of Malaria PCR▽

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**Real-time PCR assays for *Plasmodium* species utilizing universal and species-specific primers were compared to investigate variables influencing decreased assay sensitivity. Sequence variation in oligomer targets and competitive inhibition of dual-species templates in universal-primer mixes were found to decrease assay sensitivity.**

It has been estimated that at the end of 2004, 3.2 billion people lived at risk of malarial infection (20). Annually, between 350 and 500 million clinical episodes occur, with the majority being caused by *Plasmodium falciparum* or *Plasmodium vivax* (20). Of particular concern is *P. falciparum*, which causes the majority of malaria-related deaths, estimated to be between 0.7 and 2.7 million per year (2, 20). Recent increases in global human movement due to tourism, migrations, and displacement through disasters or conflict (5, 8, 12) have coincided with an increased importation of malaria into regions where the disease is not endemic (1, 7). For example, between 2002 and 2003, travelers and migrants who were born in areas of malaria endemicity constituted 19% and 18% of people moving into the United States and Australia, respectively (4, 17). In Australia, all four human *Plasmodium* species are routinely detected, with cases typically arising in migrants, travelers, soldiers, and refugees (3, 13). Traditionally, light microscopy has been utilized in *Plasmodium* species detection; however, the last decade has seen the introduction of nucleic acid amplification-based diagnostic assays (9, 18), including rapid real-time PCR methods (11, 14). Regardless of method, we emphasize that diagnostic tools need to be able to adequately detect and distinguish the malarial species, particularly the highly pathogenic *P. falciparum*. The requirement for adequate discrimination is further necessitated by the high incidence of mixed-species malarial infections (up to 12%) (6, 10, 15). In this study, we utilized published real-time PCR methods to highlight the potential for sequence variation and competitive inhibition to produce false-negative results by *Plasmodium* species PCR methods.

Two sets of real-time PCR assays targeting the plasmodium 18S rRNA gene were compared for the detection of *P. falciparum*, *P. vivax*, and *Plasmodium ovale*. Each of the real-time PCR sets comprised three separate TaqMan PCR assays for the detection of each of the three species. The first set (Peran-TM, where TM refers to TaqMan) utilized primers and probes previously described by Perandin et al. (11), while the second set (Rouge-TM) used primer and probe sequences described by Rougemont et al. (14). There were two key differences between the Peran-TM and Rouge-TM methods. First, each PCR targeted different sequences on the 18S gene of each species. Second, the Peran-TM methods used species-specific primers and probes, whereas the Rouge-TM methods used species-specific probes with a single set of universal primers for amplification of all three *Plasmodium* species. Briefly, in our study, 119 blood specimens (with labeling preserving donor anonymity) were obtained from immigrants from areas of malaria endemicity and from subjects who returned from overseas travel to areas of malaria endemicity and presented with clinical features suggestive of malaria. Nucleic acids were extracted from each specimen using the High Pure viral nucleic acid kit (Roche Diagnostics, Australia) by following the manufacturer's protocol. The original reaction conditions were modified; PCR was performed using 25-μl reaction mixtures containing 5 μl of nucleic acid extract, 10 pmol of each primer, 4 pmol of each probe, and 12.5 μl of QIAGEN QuantiTect Probe PCR master mix (QIAGEN, Australia). PCR cycling was performed on a RotorGene 3000 cycler (Corbett Life Science, Australia), with an initial activation at 95°C for 10 min and 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Of the 119 specimens tested, 108 provided agreement between the results of the Peran-TM and Rouge-TM assays; 40 specimens were positive for *P. falciparum*, 50 were positive for

TABLE 1. Sequence alignments illustrating mismatches with assay oligonucleotides

| Oligonucleotide or sample (GenBank accession no.) | Sequence[a] |
|---|---|
| Peran-TM *P. ovale* probe | CCTTTTCCCTATTCTACTTAATTCGCAATTCATG |
| Sample 15 (DQ845247) | CCTTTTCCCT**T**TTCTACTTAATTCGC**T**ATTCATG |
| Peran-TM *P. ovale* forward primer | TTTTGAAGAATACATTAGGATACAATTAATG |
| Sample 15 | TTTTGAAGAATA**T**ATTAGGAT**G**CA**T**T**A**TAGT |

[a] Mismatched bases are underlined.

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TABLE 2. Peran-TM assay $C_T$ values and Rouge-TM assay results for the 14 specimens containing both *P. falciparum* and *P. vivax* nucleic acids[a]

| Sample | Peran-TM $C_T$ value | | $\Delta C_T$ of Peran-TM | Rouge-TM result | |
|---|---|---|---|---|---|
| | *P. falciparum* | *P. vivax* | | *P. falciparum* | *P. vivax* |
| 1 | 33.5 (+) | 24.0 (+) | 9.5 | ND (−) | 25.3 (+) |
| 2 | 28.8 (+) | 21.1 (+) | 7.7 | ND (−) | 24.4 (+) |
| 3 | 34.3 (+) | 16.3 (+) | 18.0 | ND (−) | 20.7 (+) |
| 4 | 31.5 (+) | 22.1 (+) | 9.4 | ND (−) | 26.0 (+) |
| 5 | 33.7 (+) | 17.0 (+) | 16.7 | ND (−) | 23.6 (+) |
| 6 | 33.4 (+) | 19.4 (+) | 14.0 | ND (−) | 23.1 (+) |
| 7 | 18.7 (+) | 33.7 (+) | 15.0 | 18.4 (+) | ND (−) |
| 8 | 25.0 (+) | 32.2 (+) | 7.2 | 23.0 (+) | ND (−) |
| 9 | 23.7 (+) | 30.6 (+) | 6.9 | 22.7 (+) | ND (−) |
| 10 | 19.4 (+) | 27.4 (+) | 8.0 | 17.5 (+) | ND (−) |
| 11 | 22.5 (+) | 23.9 (+) | 1.4 | 20.8 (+) | 29.8 (+) |
| 12 | 28.8 (+) | 25.6 (+) | 3.2 | 27.6 (+) | 27.4 (+) |
| 13 | 23.6 (+) | 23.3 (+) | 0.3 | 22.3 (+) | 27.2 (+) |
| 14 | 24.3 (+) | 20.2 (+) | 4.1 | 27.9 (+) | 24.4 (+) |

[a] The success of assay detection for each species is indicated by "+" (positive) or "−" (negative). ND, not detected.

*P. vivax*, 1 was positive for *P. ovale*, 4 were positive for both *P. falciparum* and *P. vivax*, and 13 were negative by both protocols. Of the 11 specimens providing discrepant results, 10 were positive for both *P. falciparum* and *P. vivax* by the Peran-TM method but were positive for only one species by the Rouge-TM method (6 were positive for *P. vivax* and 4 were positive for *P. falciparum*). The presence of both species was confirmed for 9 of these 10 samples by utilizing a previously described nested malaria PCR assay (16). One specimen was positive for *P. ovale* by the Rouge-TM method but was negative by the Peran-TM method.

The specimen that produced a false-negative result in the Peran-TM *P. ovale* assay provided a cycle threshold ($C_T$) value of 25 cycles in the Rouge-TM *P. ovale* PCR. Thus, a low template load was not considered to be the source of the false-negative result. An amplification product was not observed upon gel electrophoresis of the Peran-TM *P. ovale* reaction mix. Sequencing of the 18S sequence of this *P. ovale* strain revealed two mismatches with the Peran-TM *P. ovale* probe and seven mismatches with the Peran-TM *P. ovale* forward primer (Table 1, sample 15). The concentration of mismatches at the 3′ end of the forward primer was thus considered to have prevented PCR amplification.

Investigation of the mixed-species specimens indicated that the relative concentrations of *P. falciparum* and *P. vivax* DNAs determined the ability to detect one or both species in the Rouge-TM assays. In the Peran-TM assays, the *P. falciparum* and *P. vivax* PCR $C_T$ values differed by 6.9 to 16.7 cycles for the specimens in which only one species was detected by the Rouge-TM assays (Table 2, samples 1 to 10). Thus, assuming that 3.3 cycles represent a 1-log difference in DNA loads, then there was at least a 100-fold difference in the concentrations of *P. falciparum* and *P. vivax* DNAs in these specimens. Notably, the particular species detected by the Rouge-TM assays correlated with species predicted to be at their highest concentrations based on the Peran-TM $C_T$ values. In contrast, the Peran-TM *P. falciparum* and *P. vivax* $C_T$ values differed by only 0.3 to 4.1 cycles in the samples in which both species were detected by the Rouge-TM methods (Table 2, samples 11 to 14).

To further investigate this phenomenon, we tested 10-fold dilutions of quantified *P. falciparum* DNA spiked with static amounts of quantified *P. vivax* DNA with both the *P. falciparum* Peran-TM and Rouge-TM methods. In brief, the results suggest that the Rouge-TM assays cannot reliably detect both species in cases where there is a >10-fold difference in the DNA loads. In contrast, the Peran-TM methods could reliably detect both species even where the relative loads differed by at least 100-fold (Table 3). Further investigation of the Rogue-TM assay involving a comparison of the spiked and unspiked serial dilutions showed reliable detection of the *P. falciparum* dilutions in the absence of a competing *P. vivax* template (Table 3). Similar Rouge-TM results were generated when *P. vivax* dilutions were tested with and without static *P. falciparum* spikes (data not shown). Furthermore, spiked and unspiked dilution sets were run in both assays under the conditions originally published in order to exclude the possibility of assay underperformance, and results comparable to the aforementioned data were observed (data not shown).

The limited capacity of the Rouge-TM methods to detect mixed-species infections was most likely due to the previously described mechanism of competitive inhibition caused by the use of universal primer sequences (19). The three Rouge-TM PCR assays used the same forward and reverse primers for the amplification of all three *Plasmodium* species. This meant that any of the species could be amplified in any of the three PCRs even though the detection of only one species was facilitated by a species-specific probe. Thus, if the concentration of DNA of one species exceeded that of the DNA of another species, then

TABLE 3. *P. falciparum* Rogue-TM and Peran-TM $C_T$ values for *P. falciparum* serial dilutions with and without inclusion of a *P. vivax* DNA spike[a]

| *P. falciparum* dilution | *P. falciparum* DNA only | | | *P. falciparum* DNA spiked with *P. vivax* DNA | | | |
|---|---|---|---|---|---|---|---|
| | Approx *P. falciparum* copy no. | Rouge-TM $C_T$ value | Peran-TM $C_T$ value | Approx *P. falciparum* copy no. | Approx *P. vivax* copy no. | Rouge-TM $C_T$ value | Peran-TM $C_T$ value |
| Neat | 10,000 | 20.9 | 20.5 | 10,000 | 100 | 21.6 | 19.4 |
| $10^{-1}$ | 1,000 | 24.2 | 24.3 | 1,000 | 100 | 24.5 | 23.0 |
| $10^{-2}$ | 100 | 27.7 | 28.1 | 100 | 100 | 29.0 | 26.3 |
| $10^{-3}$ | 10 | 31.7 | 31.3 | 10 | 100 | ND | 29.7 |
| $10^{-4}$ | 1 | 36.9 | 34.5 | 1 | 100 | ND | 32.2 |
| $10^{-5}$ | 0.1 | ND | ND | 0.1 | 100 | ND | ND |

[a] ND, not detected.

the DNA at the greater concentration would amplify first and subsequently monopolize the PCR.

Overall, the above results highlight the impact that sequence variation and competitive inhibition can have on the success of malaria PCR assays. However, these considerations can be applied universally in the development of any nucleic acid amplification method. Specifically, when developing and using malaria nucleic acid amplification methods, laboratories both in areas where malaria is endemic and in areas where it is not endemic need to consider the importance of identifying mixed-species infections, as well as the need for the careful design and evaluation of primers and probes. Given the above-mentioned findings, we underline the importance of utilizing conserved species-specific oligonucleotides for the PCR detection of *Plasmodium* species.

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